

EXHIBIT A

M-CSF: HAEMATOPOIETIC GROWTH FACTOR OR INFLAMMATORY CYTOKINE?

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Macrophage colony-stimulating factor (M-CSF), initially described as a growth factor of the mononuclear phagocytic lineage, also participates in immunological and inflammatory reactions, bone metabolism and pregnancy. All its biological activities are mediated by a tyrosine kinase receptor (M-CSF-R) that is encoded by the *c-fms* protooncogene. After a brief overview on the synthesis, structure, metabolism and signalling of M-CSF and its receptor, we present with more details the major in vitro and/or in vivo biological activities of this cytokine. A particular attention has been devoted to the results suggesting that the various M-CSF isoforms (i.e. soluble, cell-associated and matrix anchored forms) play different specific roles on target cells bearing M-CSF-R at their surface. Infectious, inflammatory and neoplastic diseases in which M-CSF is involved and could participate to their physiopathology are mentioned. Finally, the role that the various isoforms of M-CSF could play in the regulation of "physiological and pathological cytokine networks" during inflammation and immune responses is discussed.

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Macrophage colony-stimulating factor (M-CSF or CSF-1) is an haematopoietic growth factor that stimulates the survival, proliferation, differentiation and several functions of cells from the mononuclear phagocytes lineage.¹ It also plays a role in bone metabolism, fertility, pregnancy and inflammatory processes. A unique gene encodes several alternatively spliced mRNAs that generate three different mature dimeric proteins.² The cell surface-associated (glycoprotein) and extracellular matrix-anchored (proteoglycan) forms of M-CSF act locally while the secreted soluble (glycoprotein) M-CSF acts by humoral route, all through a specific cell surface tyrosine kinase receptor identical to the *c-fms* protooncogene product.^{2,3} We present here a brief review on the synthesis, the structure, the major biological functions and the involvement in pathology of an haematopoietic growth factor (M-CSF) that is also a cytokine.

STRUCTURE AND SYNTHESIS OF M-CSF AND ITS RECEPTOR

Structure and synthesis of M-CSF

The human M-CSF gene spans about 21 kilobases (kb) on the short arm of the chromosome 1 at position p13-p21.⁴ It contains ten exons and nine introns, the coding sequences being located in the first eight exons.^{1,2} Five cDNA have been cloned from mature alternative transcripts (mRNA) ranging in size from 1.5 kb to 4.4 kb.^{2,5} This heterogeneity is due to various combinations of a differential splicing of the coding exon 6 and of the 3' non-coding region (exons 9 and 10) of the M-CSF gene. The 5' and 3' portions (common to all cDNAs) of exon 6 encode two N-glycosylation sites and a transmembrane domain while the differentially spliced central portion encodes two intracellular proteolytic cleavage sites, a site of glycosaminoglycan addition, a site of O-glycosylation and two other sites of N-glycosylation.² The alternative splicing of non-coding exons 9 or 10 modifies the stability of the mature mRNAs. Combined with complex co- and post-translational modifications, these mRNAs generate different mature M-CSF isoforms: (1) an homodimeric secreted M-CSF glycoprotein of 85 kDa, (2) homodimeric or heterodimeric M-CSF proteoglycans (PG-M-CSF) up to 150 kDa; and (3) a cell surface-associated homodimeric glycoprotein slowly released as a soluble molecule of 44 kDa. These isoforms probably differ in their production sites and

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regulation of production as suggested by the different ratios of secreted, cell-associated and stroma-anchored M-CSF found in different cell types and under various conditions of stimulation. They act on cells expressing *c-fms* by direct cell-to-cell interactions or by autocrine, paracrine or endocrine routes.

Synthesis and structure of the M-CSF receptor (M-CSF-R)

M-CSF acts on its target cells by binding to a single class of high affinity transmembrane receptors encoded by the *c-fms* protooncogene (located on chromosome 5 at q33.3 and linked in tandem with the type β PDGF-Receptor gene).^{3,6} The 22-exons *c-fms* gene that spans 75 kb in length, consists of 21 coding exons (Exons E2 to E22) smaller than 0.3 kb separated by introns of heterogeneous size (6.3 kb to less than 0.1 kb).⁷ The non-coding exon E1 is located 26 kb upstream from the coding sequences. The transcription of the *c-fms* gene is regulated by two different tissue-specific promoters.⁶ The first one is active in placental cells, but not in haematopoietic cells. The second one, located 0.55 kb upstream of the second-coding-exon E2 is active in macrophages but not in trophoblasts. The glycoprotein *c-fms*, a ligand-inducible protein tyrosine kinase (PTK), belongs to the receptor subfamily III (including the receptors of EGF, PDGF, Insulin and IGF-1, *c-kit* and *flk2/flt3*). It consists of a single transmembrane domain which separates the extracellular part (ligand binding domain) containing five immunoglobulin repeats from the intracellular tyrosine kinase domain composed of two parts flanking a non-catalytic insertion sequence, the kinase insert.⁶

PRODUCTION, CATABOLISM AND SIGNALLING OF M-CSF

M-CSF concentrations in biological fluids and culture media can be measured by bioassays and immunoassays.⁸ M-CSF is produced in vitro by numerous cell lines spontaneously or after induction. Normal fibroblasts, endothelial cells, thymic epithelial cells, monocytes-macrophages, marrow stromal cells, B and T cells, osteoblasts, astrocytes, microglia, neurons and keratinocytes produce M-CSF in vitro either constitutively (at low levels) or after induction.^{1,8,9} Interferon γ (IFN- γ), tumour necrosis factor (TNF)- α and - β , interleukin 1 (IL-1), IL-3, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce M-CSF production by monocytes, endothelial cells, fibroblasts, T cells and polymorphonuclear leukocytes.^{1,8} In vivo, cytotrophoblasts, epithelial and glandular cells of the pregnant uterus, endothelial cells from high endothelial venules, most

fibroblasts, some lymphocytes and interdigitated reticular cells of hyperplastic and Hodgkin's disease lymph nodes produce M-CSF.^{8,10} In vitro, M-CSF binds to M-CSF-R at the surface of macrophages, the complex being rapidly degraded after endocytosis (downmodulation). In vivo, this mechanism assumes 95% of the clearance of the circulating M-CSF (half-life of 10 min) by hepatic Kupffer cells and splenic macrophages,¹¹ whereas kidneys play a minimal role.¹² M-CSF acts on cells of the monocyte-macrophage lineage including microglia, osteoclasts and on trophoblastic cells. Its binding to *c-fms* generate receptor dimerization, autophosphorylation and activation of its tyrosine kinase activity. Phosphorylated cytoplasmic secondary substrates induce a cascade of biochemical events leading to cellular responses: mitosis, secretion of cytokines, membrane ruffling,^{1,6} and regulation of transcription of its own receptor.¹³

BIOLOGICAL ACTIVITIES OF M-CSF

Haematopoiesis

M-CSF alone stimulates the growth of murine bone marrow monocytic progenitors,¹ but it also synergizes with earlier cytokines (IL-1, IL-3, IL-6) to generate large colonies from primitive progenitors such as mouse or human high proliferative potential colony-forming cells (HPP-CFCs) and purified human CD34⁺ cells.^{14,15} In addition, anti-M-CSF-R antibodies inhibit the proliferation of day-12 colony forming Units-Spleen (CFU-S) in mouse.¹⁶

These M-CSF effects seem to be dose dependent. Thus, the addition of low amounts of M-CSF to human CD34⁺ cells stimulated by IL-3 promotes the development of various types of large colonies containing immature monocytes. In contrast, the addition of high concentrations of M-CSF generates smaller and mature macrophage colonies. This could be due to a rapid down-regulation of the *c-fms* mRNA and protein by these high concentrations of M-CSF.¹³ However, a flow cytometric analysis of CD34⁺ subsets evidences that the most primitive population (CD34⁺ CD38^{lo}, CD50⁺) did not express the M-CSF-R.¹⁷ Thus, while a direct effect of M-CSF on the proliferation of late monocytic and earlier granulomonocytic committed progenitors may be assumed, its biological role on the proliferation and differentiation of primitive progenitors remains controversial. M-CSF also acts indirectly on haematopoiesis by stimulating the macrophagic production of numerous activating and inhibitory cytokines such as G-CSF, IFN, TNF and IL-1.^{8,18} In addition, M-CSF was recently identified as a growth factor for murine primary stromal initiating cells (SICs).¹⁹ These M-CSF-induced SICs that do not seem

to belong either to the macrophage lineage nor to the bone marrow CFU-F population, are defined by their ability to support the proliferation of B and myeloid lineage cell lines.¹⁹ These results suggest that M-CSF, together with other cytokines, could participate to the establishment of a functional haematopoietic micro-environment, at least in vitro. By contrast, addition of recombinant human M-CSF (rhM-CSF) to human long-term cultures inhibits haematopoiesis by inducing the production of inhibitory molecules in culture supernatants.²⁰ Thus, the effects of M-CSF on in vitro haematopoiesis are complex, and probably depend of various parameters such as the state of cell activation, the local concentration of M-CSF and the isoform produced.

Immunological defences

In vitro, M-CSF is necessary for the survival of mature monocytes/macrophages, and for several of their biological functions.¹⁸ M-CSF activates both in vitro and in vivo the anti-bacterial and anti-fungal activities of macrophages. It increases their phagocytic capacity, their production of reactive oxygen intermediates, and their killing capacities against various micro-organisms.^{18,21} In humans, clinical studies suggest its usefulness as an adjunctive therapy in patients with invasive fungal disease.²¹ Tumoral cells and tumour-associated macrophages (TAMs), present in the stromal environment of most tumours, stimulate each other to produce cytokines and various molecules that can favour either the growth of the primary tumour and metastases or the tumoricidal activity of macrophages.²² M-CSF locally produced by tumoral cells,⁸ is a chemotactic factor favouring tumour infiltration by monocytes.^{22, 23} It also renders macrophages responsive to a secondary signal [such as lipopolysaccharide (LPS) and membrane phospholipids] that triggers their immunological functions mediated by the secretion of molecules such as TNF- α .²⁴ Interestingly, Jadus *et al.*²⁵ demonstrated that freshly isolated macrophages kill only tumoral cells expressing the membrane isoform of M-CSF. The authors suggest that macrophages received at the same time the priming (membrane M-CSF) and a secondary triggering signal delivered by other membrane molecules specific to tumoral cells, since activated macrophages do not kill normal cells.²⁵

Bone metabolism, osteoclasts and tissue macrophages

The congenital osteopetrosis of *op/op* mice is due to an inactivating mutation in the M-CSF gene that results in the total absence of biologically active M-CSF.²⁶ These toothless mice with increased and densified bone mass have a total absence of osteoclasts explaining the absence of physiological bone resorp-

tion. Repeated injections of M-CSF to *op/op* mutant mice correct their osteopetrosis and allow teeth eruption and growth, demonstrating that M-CSF is a major growth factor for osteoclasts. The proliferation and differentiation of osteoclast progenitors (derived from CD34⁺ haematopoietic cells together with monocyte progenitors), the osteoclastic functions of mature osteoclasts, their migration and their survival are modulated by M-CSF, in co-operation with other soluble factors and cell-to-cell interactions provided by osteoblasts and fibroblasts. Recently, a specific role for the locally produced soluble, matrix anchored and membrane bound isoforms of M-CSF in the balance of these processes has been suggested.²⁷ The *op/op* mice have also a 10-fold reduction of blood monocytes and an absence of tissue macrophages in several locations. Injections of M-CSF to *op/op* animals restore a normal number of blood monocytes, bone marrow and splenic macrophages and of Kupffer cells (that is normal at birth, but decrease postnatally).²⁶ By contrast, systemic administration of M-CSF is ineffective to restore the number of pleural and peritoneal macrophages, except by local injection. Finally, dermal, thymus, lymph node antigen presenting cells and bone marrow monocytes are not altered in *op/op* mice and not modified by M-CSF injections, confirming that these cells depend on other growth factors such as GM-CSF.^{26, 27} These results suggest a specific role of the different isoforms of M-CSF in the development and functions of the various macrophage populations in vivo.

Pregnancy

The progressive increase of M-CSF serum levels during pregnancy in women and mice correlates with a local production by maternal uterine epithelial cells and trophoblasts induced by oestrogens and progesterone.²⁸ In the same time, placental trophoblasts express M-CSF-R. In pregnant mice, the major M-CSF transcript (2.3 kb in length) in uterine epithelial cells has the full length exon 6 and the 3' end exon 9 (devoid of AU-rich sequence that confers mRNA instability).²⁹ In pregnant women, a 3.0-kb M-CSF mRNA (short exon 6 and 3' end exon 10) generates a membrane-bound M-CSF that is responsible for a paracrine effect on placental cells bearing M-CSF-R, and of a massive local concentration of M-CSF favouring monocyte recruitment.³⁰ The female *op/op* mice have a reduced fertility rate,³¹ while *op/op* males are completely fertile when mated to heterozygous females. This strain then represents a valuable natural knock-out model for exploring the role of M-CSF in fertility and pregnancy. Pollard *et al.* showed that *op/op* females mated with *op/op* males are infertile while they become pregnant (at a ratio of 46% of control females) when mated with *+ / op* males. They suggest that M-CSF, while not mandatory for placental growth, plays an important

role in ovulation, implantation, and survival of embryos. Recently, another group has found evidence that M-CSF and macrophages are involved in folliculogenesis and ovulation.³² Variations of M-CSF levels in woman during human pregnancy and after hormonal ovarian hyperstimulation,³³ are very similar to those described in mice,²⁸ suggesting that abnormal M-CSF production or abnormal ovarian or uterine responses could be responsible for some sterilities.

Others effects

In vivo injections of M-CSF in both human and animals result in modifications of circulating white blood cells: lymphocytes and platelets significantly decrease while monocytes and polymorphonuclear cells increase.⁸ The increase of M-CSF serum levels during pregnancy was accompanied by similar blood modifications.³³ M-CSF also plays a role in the clearance and metabolism of lipoproteins and cholesterol by macrophages in human and rabbits, that could be useful for the treatment of some hyperlipaemias.³⁴ Oxidized lipoproteins involved in the genesis of atherosclerosis induce the production of M-CSF by endothelial and smooth muscle cells.³⁵ Thus, M-CSF might recruit and activate macrophages to infiltrate and participate to the formation of organized atherosclerotic plaques.^{34,35} M-CSF induces *in vitro* the proliferation of microglial cells that derive from the monocyte lineage.³⁶ Its biological role in the central nervous system (CNS) *in vivo* is not yet investigated in *op/op* mice.

IS M-CSF INVOLVED IN THE PHYSIOPATHOLOGY OF MALIGNANT AND INFLAMMATORY DISEASES?

Several mutated forms of the *c-fms*/M-CSF-R are oncogenic when transfected in haematopoietic cells or fibroblasts. The expression of both the ligand and the receptor generate their *in vitro* transformation by autocrine growth but not always *in vivo* tumorigenicity.^{6,8} While freshly isolated blasts of acute myeloid leukaemia (AML) express *c-fms* in many cases and M-CSF in some cases,³⁷ an autocrine loop has never been described. Oncogenic mutations of *c-fms* are found rarely in AML and myelodysplasias.³⁸ When activated by M-CSF, the M-CSF-R present at the surface of hairy cells in Hairy Cell Leukaemia induces chemotactic movements without affecting their proliferation.³⁹ In combination with other differentiating agents (vitamin D₃ and 12-O-tetradecanoylphorbol-13-acetate), M-CSF induces the macrophage terminal differentiation of an acute promyelocytic leukaemia cell line, suggesting its potential interest in therapeutic assays to induce terminal differentiation of leukaemic cells.⁴⁰ Finally, the elevated M-CSF serum levels

in patients with several haematological pathologies⁴¹ might be used as a diagnostic, prognosis or follow up marker during therapy.⁴² In ovarian and breast cancer, M-CSF serum levels correlate with prognosis, activity and invasiveness of the disease.⁴³ An abnormal overexpression of M-CSF and *c-fms* (mRNA and proteins) is detected in primary tumours and tumour cell lines of epithelial origin.^{43,44} It is proposed that the M-CSF, locally produced by breast tumoural cells and tumour-infiltrating cells, could activate TAMs to produce cytokines and enzymes that activate tumour proliferation, invasion and metastasis.^{23,43,44} On the opposite, other results (mentioned above; Ref. 25) show that M-CSF enhances the anti-tumour activity of TAMs. These contradictory results suggest that these opposite effects are dependent on the balance between the different isoforms of M-CSF around tumour cells and able to trigger TAMs.⁴⁴ M-CSF, known to favour HIV replication in monocytes, is produced intracerebrally by HIV-infected monocytes and activated astrocytes, explaining its elevated cerebrospinal fluid levels in HIV patients.⁴⁵ M-CSF locally produced may be responsible of the proliferation and activation of microglial cells that release cytokines, cytotoxins and neurotoxins involved in HIV and other inflammatory or immunological induced CNS damage.^{36,45}

During inflammation and infection, an early elevation of blood and tissue M-CSF concentrations is later followed by an increase of activated macrophages with immunosuppressive activity.^{12,21,46} We suggest that in inflammatory processes the early local production of M-CSF (by T and/or other cells), stimulates blood monocytes to migrate and to activate the local defences. In a second period, resident macrophages permanently activated (by M-CSF and other cytokines) could allow the physiological slow-down and disappearance of inflammatory reaction once the triggering agent has been eliminated. In some cases, genetic or environmental reasons could establish and maintain a "pathological cytokine network" that initiates an inflammatory or dysimmune disease. The isoforms of M-CSF present locally and the kinetics of their appearance during physiological and pathological reactions has still to be explored.

CONCLUSION

M-CSF, first identified as a haematopoietic growth factor specific of monocytic cells, is a cytokine active on other cell types and tissues. The precise roles and mechanisms of action of M-CSF *in vivo* still remain rather ignored, even if the *op/op* mouse model brought some answers to numerous questions. M-CSF induces macrophages to release other cytokines triggering other cell types to produce other signals,

in vitro and in vivo investigations are complex. In the last few years the identification of various isoforms of M-CSF that could play specific roles has emerged as one of the most interesting aspects for understanding the biological role of M-CSF in physiology and pathology.

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